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^{1}H Nuclear Magnetic Resonance Study of Oxazolidinone Binding to Bacterial Ribosomes

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The oxazolidinones are a novel class of antibiotics that inhibit initiation of protein synthesis in bacteria. In order to investigate their novel mechanism of action, the interactions of several oxazolidinones with bacterial 70S ribosomes, 50S subunits, and 30S subunits have been characterized by ^{1}H nuclear magnetic resonance (NMR) line-broadening analyses and transferred nuclear Overhauser enhancement (TRNOE) experiments. PNU-177553 and PNU-100592 (eperezolid) and their corresponding enantiomers, PNU-184414 and PNU-107112, were studied. The dissociation constants were determined to be 94 ± 44 μM and 195 ± 40 μM for PNU-177553 and eperezolid, respectively. There was a ~4-fold decrease in affinity for their corresponding enantiomers. The NMR-derived dissociation constants are consistent with their antibacterial activity. PNU-177553 and eperezolid were found to bind only to the 50S subunit, with similar affinity as to the 70S ribosome, and to have no affinity for the 30S subunit. Specific binding of PNU-177553 was further confirmed in TRNOE experiments in which positive NOEs observed for the small molecule alone were changed to negative NOEs in the presence of bacterial 70S ribosomes. The observed NOEs indicated that PNU-177553 did not adopt a significantly different conformation when bound to the 70S ribosome, compared to the extended conformation that exists when free in solution. Since this is likeliest the case for each of the four compounds included in this study, the A ring C5 side chain may be positioned in the proper orientation for antibacterial activity in PNU-177553 and eperezolid but not in their inactive enantiomers.

The oxazolidinones represent a new chemical class of antibacterial agents with activity against gram-positive organisms (5, 6). It has been demonstrated that the oxazolidinones linezolid and eperezolid are active against methicillin-resistant *Staphylococcus aureus*, penicillin-resistant *Streptococcus pneumoniae*, and vancomycin-resistant *Enterococcus faecium* (6, 8, 9, 11, 20). It has been shown previously that the oxazolidinone eperezolid binds to the 50S ribosomal subunit and competes with binding of chloramphenicol and lincomycin (10). However, the oxazolidinone mechanism of action is clearly distinct from these and other antibiotics. Studies to precisely define this mechanism are ongoing. Recently, it was demonstrated that the oxazolidinone linezolid inhibits formation of the initiation complex in bacterial translation systems by preventing formation of the *N*-formyl-methionyl-tRNA-ribosome-mRNA ternary complex (17). In order to investigate their novel mechanism of action, the interactions of several oxazolidinones with bacterial 70S ribosomes, 50S subunits, and 30S subunits have been characterized by ^{1}H nuclear magnetic resonance (NMR) line-broadening analyses and transferred nuclear Overhauser enhancement (TRNOE) experiments. The structures of the oxazolidinones studied, PNU-177553 and PNU-100592 (eperezolid), and their corresponding inactive enantiomers, PNU-184414 and PNU-107112, are shown in Fig. 1.

Eperezolid binding to the 50S ribosomal subunit has previously been studied using radiolabeled [^{14}C]eperezolid (10). A Scatchard plot indicated a K_d of ~20 μM for this interaction. The relatively large scatter of the data in the Scatchard plot for this weak interaction and the requirement for radiolabeled compounds preclude the use of this technique to analyze a series of oxazolidinones. However, the weak interaction of oxazolidinones with the ribosome can be ideally monitored by NMR spectroscopy using unlabeled compounds. The weak binding interaction results in fast chemical exchange on the NMR time scale. The high molecular weights of the 70S ribosome or the 30S and 50S subunits result in an easily observable line broadening of the oxazolidinone resonances if an interaction exists. Titration experiments provide a quick method of determining the binding constant. Weak binding also allows the use of TRNOE experiments to probe the interaction (13). In a TRNOE experiment, the small-molecule ligand is present in large excess over the macromolecule. During the mixing time of the experiment, the ligand molecules exchange between their bound and unbound states. However, since NOEs build up much faster in the bound state because of the longer correlation time associated with the macromolecule, the resulting NOESY spectrum contains information regarding the bound conformation of the ligand. The sign of the NOE is also inverted from that for the ligand in the absence of macromolecule. TRNOE studies have been used previously to study macrolide-ribosome interactions (1–4, 7). The bound conformation of macrolides, such as erythromycin, erythromycin analogues, roxithromycin, and ketolides, has been determined. We have applied the technique here for the first time to study the interaction of oxazolidinones with ribosomes.

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MATERIALS AND METHODS

Preparation of *Escherichia coli* 70S ribosomes. *E. coli* MRE600 (ATCC 29417) was grown in Lennox L broth (Gibco BRL, Gaithersburg, Md.) at 37°C. Ribosomes were prepared by the low-salt wash method of Rheinberger et al. (15).

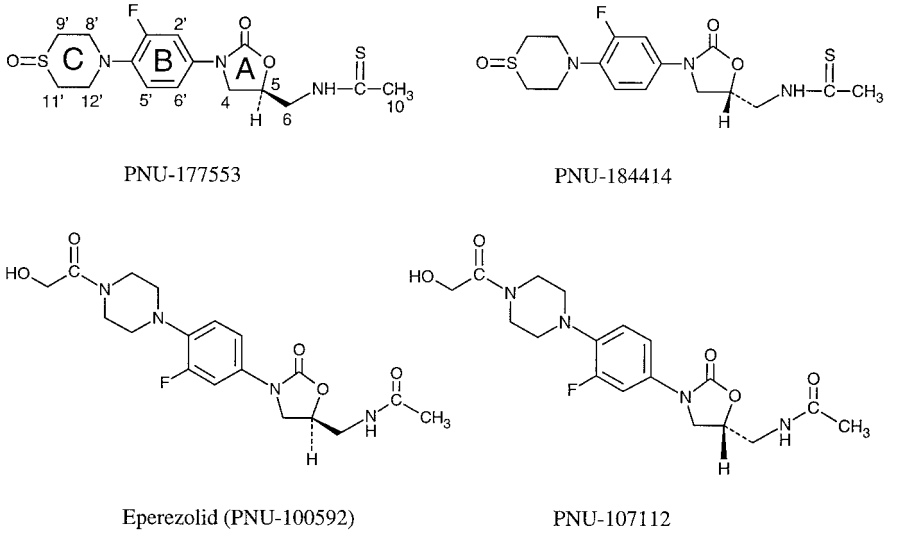

FIG. 1. Chemical structures of PNU-100592 (eperezolid), PNU-107112, PNU-177553, and PNU-184414. The three rings are designated A, B, and C, respectively, from right to left. The ^{1}H numbering scheme used for PNU-177553 is indicated.

Fifty grams (wet weight) of frozen MRE600 cells was mixed with an equal weight of alumina, and the cells were lysed at 0°C by grinding with a mortar and pestle. Fifty milliliters of buffer A (10 mM Tris-HCl [pH 7.4], 30 mM NH_4Cl, 10 mM $MgCl_2$, and 1 mM dithiothreitol) containing 1 μg of DNase (RNase-free; Worthington, Freehold, N.J.) per ml was added, and the suspension was stirred for 20 min. The alumina, unbroken cells, and cellular debris were removed by two centrifugations at 10,000 × *g* for 10 min. The supernatant was centrifuged again for 30 min at 30,000 × *g* (S30 extract), and the upper two-thirds of the resulting supernatant was centrifuged again at 100,000 × *g* for 16 h (S100 extract). The ribosome pellet was suspended in buffer B (10 mM Tris-HCl [pH 7.4], 1 M NH_4Cl, 1 mM $MgCl_2$, and 1 mM dithiothreitol) and centrifuged at 10,000 × *g* for 10 min, and the clear supernatant was centrifuged at 105,000 × *g* for 4 h. The pelleted ribosomes were then resuspended in a small amount of buffer A and loaded onto a 10 to 40% continuous sucrose gradient and centrifuged for 16 h at 100,000 × *g*. The fraction of the gradient containing 70S particles was pelleted as above, resuspended in buffer A at 70 to 120 mg of ribosome per ml, and stored at −80°C until needed.

Preparation of *E. coli* ribosomal subunits. Ribosomal subunits were prepared as described by Staehelin and Maglott (16), with the following modifications. The S30 extract was prepared as described above by using MRE600 mid-logarithmic-phase cells. Buffers A, B, and C were as described above except that they contained only 1 mM $MgCl_2$. The 50S and 30S ribosomal subunits were purified using a 10 to 40% sucrose gradient.

NMR sample preparation. Ribosomes were thawed out at room temperature and then held at 37°C for 5 min to activate them. A predetermined amount was then added to buffer solution consisting of 100 mM phosphate and 100 mM KCl at pH 7.0 in ^2H_2O. The oxazolidinone compounds were first dissolved in ^2H_2O. A predetermined amount of the stock solution was then lyophilized to prepare solid material which was added directly to the assay solution.

NMR spectroscopy. All NMR spectra were recorded at 27°C on a Bruker DRX-500 spectrometer using a 5-mm triple-resonance probe with 3-axis gradients. Proton chemical shifts were referenced to the ^1H_2O signal at 4.70 ppm (tetramethylsilane = 0 ppm). WATERGATE (14) was used to eliminate the water resonance in one-dimensional ^{1}H NMR spectra, while continuous-wave, low-power saturation was applied during the relaxation delay to attenuate the residual water resonance in two-dimensional NOESY experiments. The mixing time was varied from 150 to 600 ms, with 300 ms found to result in the highest-quality spectra. Typical two-dimensional data sets consisted of a data matrix of 1,024 by 128 complex points, with 64 scans collected for each increment. The sweep width in both dimensions was 8,012 Hz. The indirect dimension was zero filled during processing.

Data analysis. A line shape analysis of the ^{1}H NMR resonances of the oxazolidinone compounds in the presence of ribosomes was carried out (19). Chemical exchange in the system can be described by the simple model $E + L \rightleftarrows EL$.

If this exchange proceeds rapidly on the NMR time scale, the dissociation constant, $K_d = k_{-1}/k_1$, can be estimated by monitoring the chemical shift or line width change as a function of ligand (L) and macromolecule (E) concentration. When the bound ligand fraction, f_{EL}, is small compared to total ligand (f_L), the observed NMR parameter P_{obs} (chemical shift, transverse relaxation time, or line width) can be expressed in terms of apparent values for the bound state (P^*_{EL}) as $f_L P_L + f_{EL} P^*_{EL}$, with $f_L + f_{EL} = 1$. From the equilibrium equation and $[L] \gg [EL]$, where $L_t \approx [L]$,

$$P_{obs} = E_t(P^*_{EL} - P_L)/(K_d + L_t) + P_L \quad (1)$$

If the condition $[L] \gg [EL]$ is not satisfied, $[L]$ can be calculated using an estimated K_d. A plot of $L_t(P_{obs} - P_L)$ versus $[L]$ can be used to correct the error due to the difference of $[L]$ and L_t. This process can be iterated several times until K_d does not change:

$$L_t(P_{obs} - P_L) = E_t[L](P^*_{EL} - P_L)/(K_d + [L]) \quad (2)$$

In the experiments described here, the ribosome concentration was held constant while the ligand concentration was increased. The line width of selected resonances was then measured at each concentration of ligand. From the best fit to equation 1 or 2, the dissociation constant K_d was assessed.

RESULTS

Line width analyses to assess binding affinity of oxazolidinone compounds to 70S ribosome. The line width of the eperezolid resonance at 7.1 ppm was carefully measured at a series of eperezolid concentrations while the ribosome concentration was held constant. The line width as a function of eperezolid concentration is shown in Fig. 2. The solid line represents the best fit to equation 1. The calculated $K_d = 195 \pm 40$ μM. The enantiomer of eperezolid, PNU-107112, was also examined in this fashion with $K_d = 761 \pm 85$ μM. For PNU-177553 and its enantiomer, PNU-184414, K_ds were determined to be 94 ± 44 μM and 474 ± 285 μM, respectively.

Oxazolidinones bind to the 50S and not to the 30S ribosomal subunit. In a previous study using radiolabeled [^{14}C] eperezolid (10), it was found that eperezolid binds to the 50S ribosomal subunit. This fact was confirmed by ^{1}H NMR line width analyses of eperezolid in the presence of 50S and 30S subunits as shown in Fig. 3. There is a large line width change as a function of eperezolid concentration in the presence of the 50S subunit but only a barely appreciable line width change in

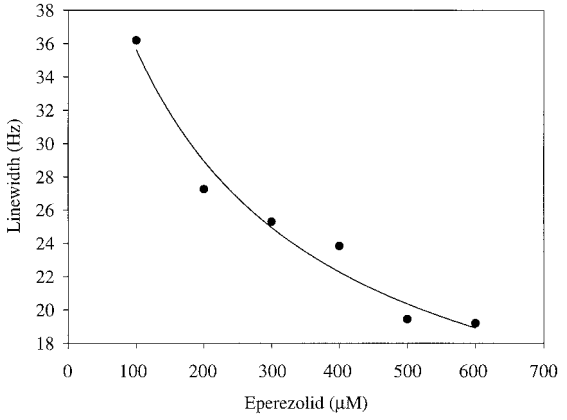

FIG. 2. Line width of the eperezolid resonance at 7.1 ppm as a function of concentration in the presence of 20 μM *E. coli* 70S ribosome. The solid line represents the best fit to equation 1 with K_d = 195 μM.

the presence of the 30S subunit. The K_d values for the 50S subunit were obtained from fitting the data by either equation 1 or 2, the latter to correct the error caused by the relatively high subunit concentration. For eperezolid and its enantiomer PNU-107112, K_d values were determined to be 201 ± 49 μM and 707 ± 103 μM, respectively. For PNU-177553, K_d was determined to be 124 ± 12 μM. The affinity of the oxazolidinones for the 50S subunit is similar to that determined for whole 70S ribosomes. There is only extremely weak affinity for the 30S subunit, and it was not possible to quantitate the dissociation constants from NMR data. Since any interaction with the 30S subunit is most likely nonspecific, the 30S subunit provides a control for nonspecific binding. Interactions observed with the 50S subunit and 70S ribosomes are higher-affinity, specific interactions.

TRNOE study to determine conformation of ribosome-bound oxazolidinones. TRNOE experiments were carried out to characterize the ribosome-bound conformation of PNU-177553. At a 680:1 ratio of PNU-177553:70S ribosomes, the sign of the cross-peaks arising from PNU-177553 changed from negative (positive NOE) to positive (negative NOE) relative to diagonal peaks as shown in Fig. 4. The negative NOEs observed can provide information regarding the bound conformation. There was no observable NOE between the methyl group protons and protons in the A ring. This end of the PNU-177553 molecule thus exists in an extended conformation when bound to the 70S ribosome. The C ring is symmetric, with the $^1H^{8',12'}$ axial and $^1H^{8',12'}$ equatorial proton resonances degenerate. NOEs of similar magnitude were observed between the $^1H^{5'}$ proton and both overlapping $^1H^{8',12'}$ axial and $^1H^{8',12'}$ equatorial proton pairs in either PNU-177553 alone or with added 70S ribosome. This is indicative of free rotation of this ring in both free and ribosome-bound PNU-177553. In the A ring of free PNU-177553, the two $^1H^4$ protons had the same-magnitude NOE interaction with the $^1H^{6'}$ proton. The two $^1H^4$ protons also had the same-magnitude NOE interaction with the $^1H^{2'}$ proton, but these NOEs showed only about 70% of the intensity of the NOEs with the $^1H^{6'}$ proton. This suggests that free rotation of the B ring with respect to the A ring does not occur. Similar ratios were observed for ribosome-bound PNU-177553, suggesting that the solution-preferred conformation of PNU-177553 is maintained when bound to the ribosome. In contrast to the macrolides studied by TRNOE methods (1–4, 7), PNU-177553 gave rise to much fewer NOEs because of its smaller size and extended conformation. In light of this and the free rotation observed for the C ring, we did not attempt to calculate an energy-minimized structure for ribosome-bound PNU-177553.

DISCUSSION

The NMR-derived affinity of the four oxazolidinone compounds for the 70S ribosome correlated qualitatively with their antibacterial activity. The most potent antibacterial compound studied here, PNU-177553, had an approximately twofold-higher affinity for the 70S ribosome than eperezolid, which has slightly less antibacterial activity. The enantiomers of both PNU-177553 and eperezolid, which have no antibacterial activity, had approximately fourfold-weaker interactions with the 70S ribosome. The fact that both enantiomers have some binding affinity suggests that the structural motifs common to the enantiomeric pairs of compounds account for some of the binding affinity. It is interesting that the inactive enantiomers of oxazolidinones such as PNU-107112 have very weak antibacterial activity, exhibiting MICs of >128 μg/ml, whereas the active enantiomer PNU-100592 has an MIC of 4 μg/ml for *S. aureus*. Therefore, the binding affinity of oxazolidinones for ribosomes does not truly reflect the antimicrobial potency. The high K_d values obtained in this study under native equilibrium conditions point to the absence of a factor that enhances oxazolidinone binding to the ribosome. It is clear, however, that the proper orientation of the functional group at the C5 position of the oxazolidinone A ring is critical for optimal binding and antibacterial activity.

TRNOE data on one of the compounds, PNU-177553, indicated that it did not adopt a significantly different conformation when bound to the 70S ribosome, compared to the extended conformation that exists when free in solution. Since this is likeliest the case for each of the four compounds included in this study, the A ring C5 side chain may be positioned

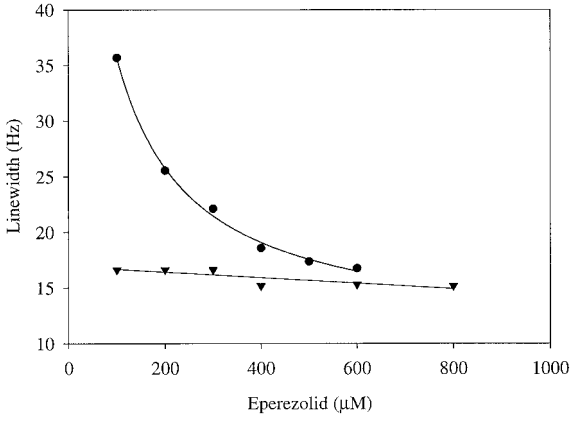

FIG. 3. Line width of the eperezolid resonance at 7.1 ppm in the presence of the *E. coli* ribosome 50S (filled circle) and 30S (filled triangle) subunits. The concentration of 50S and 30S subunits was 60 μM in assay solution.

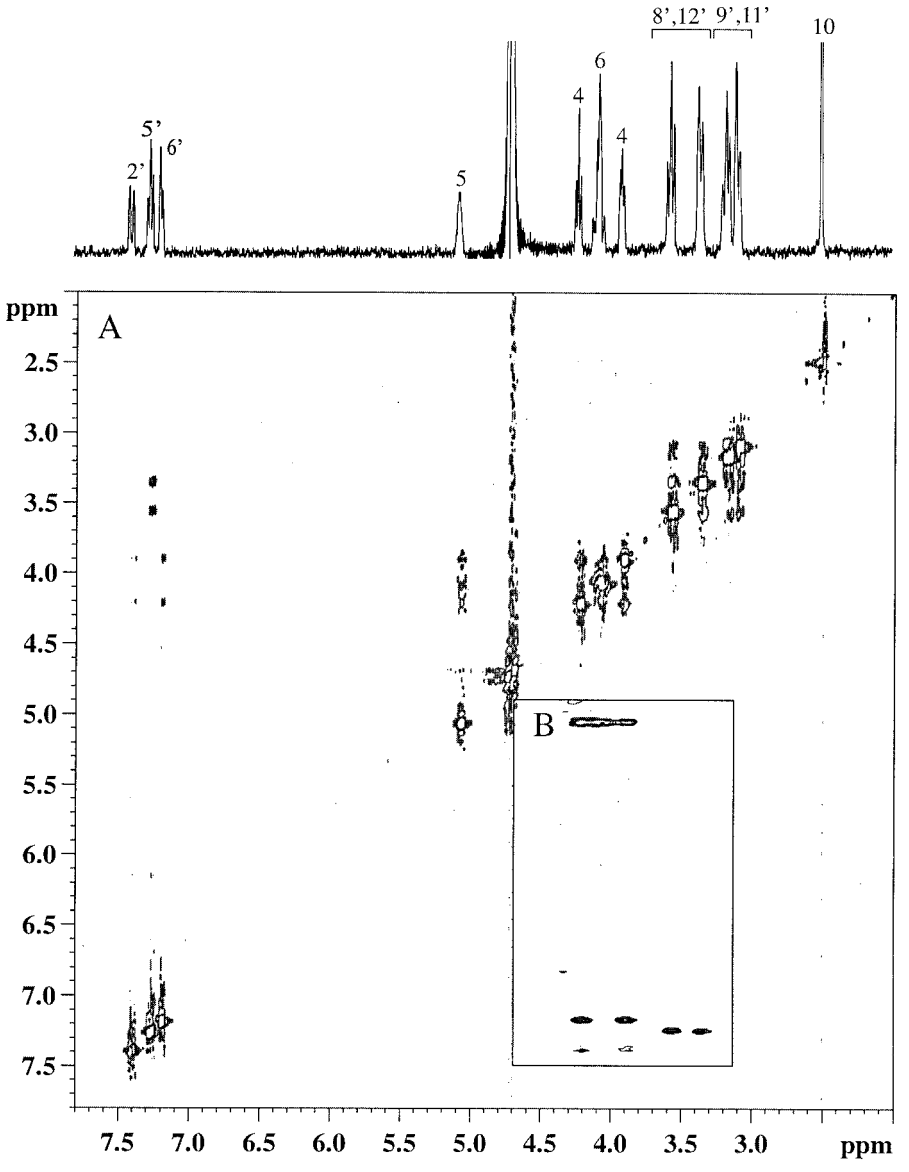

FIG. 4. NOESY contour plots for PNU-177553. (A) PNU-177553 (680 μM). (B) PNU-177553 (680 μM) with 1 μM *E. coli* 70S ribosomes. The mixing time was 300 ms. Cross-peaks are negative in panel A and positive in panel B. The one-dimensional ^{1}H NMR spectrum of PNU-177553 is shown along the top with assignments.

in the proper orientation for antibacterial activity in PNU-177553 and eperezolid but not in their inactive enantiomers.

It has been shown previously that radiolabeled eperezolid binds specifically to the 50S ribosomal subunit with a K_d of 20 μM (10). However, data collection required trapping ^{14}C-labeled eperezolid in a denatured ribosome pellet. The present study employed native conditions and ^{1}H NMR spectroscopy to measure the binding affinity of four different oxazolidinones to the ribosome under equilibrium conditions, confirming that this new class of antibiotics specifically binds to 50S and 70S but not to 30S subunits. The K_d of 195 ± 40 μM determined for eperezolid in this study was nearly 10 times greater than that obtained with the denaturation assay (10). Although this represents an order-of-magnitude difference between the two techniques, even 20 μM represents a rather high K_d for an antibiotic with the potency of eperezolid. Therefore, the interaction of eperezolid with salt-washed ribosomes is weak, regardless of the technique used to measure it. However, given the native conditions employed in the present study, the higher K_d is likely to reflect the true affinity of oxazolidinones under these ribosome and experimental conditions. The relative K_d values of the four oxazolidinone compounds for the ribosomal subunits are more important than any single K_d, since it is likely that oxazolidinone binding to the ribosome is enhanced by the presence of other players in translation (initiation factors, tRNAs, etc.). This will be the subject of a future study.

It is interesting that the oxazolidinone binding site has been probed by both UV-induced cross-linking using an azido derivative of an oxazolidinone and by chemical footprinting using dimethyl sulfate (12). In those studies, the oxazolidinone bind-

ing sites were determined to be located on both the 30S and 50S subunits, with rRNA being the only target. More recently, resistance mutations clustered in the vicinity of the central loop of domain V of 23S rRNA suggest that this rRNA region is the major site of interaction on the ribosome (18). The NMR data presented here suggest that only the 50S subunit is absolutely required for binding and that any interaction with the 30S subunit is probably indirect. Our data support the model proposed previously whereby the oxazolidinones bind to the 50S subunit first and then interact with the 30S subunit to inhibit the formation of the initiation complex in bacterial translation systems by preventing formation of the *N*-formyl-methionyl-tRNA-ribosome-mRNA ternary complex (17). A possible mechanism may be the displacement of the $tRNA_f^{Met}$ *N*-formyl-methionine backbone atoms by the oxazolidinone A ring and the C5 side chain. This could account for the stereoselectivity at C5, since only one enantiomer would mimic the $tRNA_f^{Met}$ *N*-formyl-methionine backbone atom conformation. Conservation of structure in the B and C rings would explain the observation that all four oxazolidinone compounds have some binding affinity for the ribosome.

The oxazolidinone binding site on the 50S subunit could be further localized by carrying out similar NMR titration experiments on the individual protein or nucleic acid components of the 50S subunit. Alternatively, the 50S subunit could be repeatedly fractionated until loss of binding is observed. The latter approach may have a better chance of success, since oxazolidinone binding may require more than just a single protein or nucleic acid component.

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